Stable black phosphorus/Bi$_2$O$_3$ heterostructures for synergistic cancer radiotherapy

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A R T I C L E   I N F O

Article history:
Received 2 March 2018
Received in revised form 31 April 2018
Accepted 12 April 2018
Available online 13 April 2018

Keywords:
Black phosphorus
2D Layered materials
Radiotherapy
Photodynamic effect
Cancer therapy

A B S T R A C T

X-ray induced photodynamic therapy (X-ray-PDT) is a promising approach for synergistic cancer radiotherapy and development of suitable radiosensitizers is highly desired. In this paper, we propose black phosphorus/Bi$_2$O$_3$ (BP/Bi$_2$O$_3$) heterostructures as efficient and biocompatible radiosensitizers for synergistic cancer radiotherapy. The heterostructures are synthesized by growth of ultrasmall Bi$_2$O$_3$ nanoparticles onto BP nanosheets. The Bi$_2$O$_3$ decoration inhibits the rapid degradation of BP nanosheets by occupation of the defect sites, and the synergistic effects of BP and Bi$_2$O$_3$ enable 1O$_2$ overproduction under X-ray irradiation. This X-ray-PDT effect of the BP/Bi$_2$O$_3$ nanosheets enhances the radiotherapy activity towards cancer cells by inducing cell apoptosis and cycle arrest. In vivo treatment of melanoma conducted on a clinical radiotherapeutic instrument demonstrates that the BP/Bi$_2$O$_3$ sensitized radiotherapy inhibits tumor growth efficiently. Furthermore, the BP/Bi$_2$O$_3$ nanosheets composed of biological friendly P, O, and Bi elements shows good biocompatibility in vitro and in vivo. This radiosensitizer thus has immense clinical potential for cancer therapy, and our findings reveal a general strategy to fabricate stable BP-based heterostructures for different applications.

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1. Introduction

Photodynamic therapy (PDT) is undergoing fast development as in the treatment of a variety of malignant cancers because of minimal invasiveness, low cytotoxicity, and little intrinsic or acquired resistance compared to traditional radiotherapy and chemotherapy approaches [1,2]. In the conventional PDT system, semiconductor photosensitizers have generally been used to absorb and transfer the energy of incident light to surrounding oxygen or water molecules producing reactive oxygen species (ROS). The most important species is singlet oxygen (1O$_2$) [3], which creates damage and apoptosis of nearby cancerous cells [4]. Visible or near-infrared light has generally been used in PDT, however, their penetration depth is normally less than 1 cm thus hindering clinic application of PDT to deep-seated cancers.

Owing to the strong tissue penetration ability, X-ray-induced PDT (X-ray-PDT) has aroused increasing interest [5–10], and utilization of X-ray as an energy source may enable integrated imaging, PDT, and conventional radiotherapy as the next-generation therapeutic approach [7]. Since most semiconductor photosensitizers cannot be excited by X-ray directly, heterostructures composed of semiconductors and high-Z elements (such as Au, Ag, Bi etc.) have been proposed [7–21]. The high-Z elements with large photoelectric cross sections are employed as energy transducers to indirectly excite the nearby semiconductors via energy transfer to enable efficient X-ray-PDT. In cancer radiotherapy, high-Z elements produce high-energy photoelectrons and low-energy Auger electrons efficiently hydrolyzing surrounding water molecules to produce ROS [21]. The energy transferred to the underlying semiconductor further enhanced ROS generation to produce synergistic effects.
For example, a heterostructure composed of ZnO nanorods and Au nanoparticles with enhanced ROS generation efficiency has been described [22]. Nevertheless, in spite of recent advances, the development of efficient and biocompatible radiosensitizers is still highly desired.

Black phosphorus (BP), a new 2D layered semiconductor, has emerged as rapidly rising stars in the field of nanomedicine [26–37]. As a direct bandgap semiconductor, BP possesses excellent optical properties such as photothermal, optoacoustic, and photodynamic effects [37,38]. BP nanosheets with unique 2D structure have thus been regarded as promising theranostic agents in drug delivery, imaging, and phototherapies [37–45]. Compared to other nanomaterials, BP is attractive because it is biodegradable and its degradation products are nontoxic phosphate and phosphonate. Furthermore, P is one of the essential elements in human organs and makes up about 1% of the total body weight [29]. Despite these obvious merits, the biomedical application of BP is hampered by the rapid degradation in aqueous media and to the best of our knowledge, the use of BP in radiotherapy has not been reported.

In this study, a new X-ray-PDT radiosensitizer has been proposed by growth of ultrasmall Bi2O3 nanoparticles onto BP nanosheets, obtaining BP/Bi2O3 heterostructures. By decoration with Bi2O3, the stability of BP in water is greatly enhanced and furthermore, the synergistic effects rendered by BP and Bi2O3 enable X-ray induced overproduction of 1O2. Besides, the presence of high-Z element Bi also enable to emit electrons to generate free radicals under X-ray irradiation, thus a synergistic cancer radiotherapy is realized [8]. In vitro and in vivo experiments are performed systematically to study the X-ray-PDT performances and synergistic cancer radiotherapy of the BP/Bi2O3 heterostructures.

2. Materials and methods

2.1. Materials

The BP crystals were purchased from a commercial supplier (Smart-Elements) and stored in a dark Ar glove box. N-Methyl-2-pyrrolidone (NMP, ≥99.5%) was purchased from Aladdin Ltd. (Shanghai, China). Bismuth nitrate pentahydrate (Bi(NO3)3·5H2O, 99.99%) was obtained from Sigma-Aldrich and ethylene glycol (EG, ≥99.0%) was bought from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). All the chemicals were used without further purification. Ultrapure water (18.25 MΩ cm, 25 °C) was used to prepare the solutions.

2.2. Synthesis of BP nanosheets

20 mg of bulk BP were ground, tip-sonicated for 3 h, and bath-sonicated for 5 h in 20 mL of the NMP solution. The BP nanosheets were prepared by centrifuging the exfoliated solution at 7000 r/min for 10 min and 12,000 r/min for 15 min. The precipitate was redispersed in 20 mL of the NMP solution and the concentration of BP nanosheets was about 60 μg/mL.

2.3. Synthesis of BP/Bi2O3 heterostructures

To produce the BP/Bi2O3 heterostructures, 3.5 mL of 25 mM Bi(NO3)3 in EG were added to 21 mL of 20 μg/mL BP nanosheets in NMP. The mixture was shaken and kept still for 3 h at room temperature. The mixture was then centrifuged at 12,000 r/min for 10 min to remove excess Bi(NO3)3 and the precipitate was redispersed in water for further use.

2.4. Characterization

The TEM and HR-TEM images were taken on the FEI Tecnai G2 F30 transmission electron microscope at an acceleration voltage of 200 kV and SEM and EDS were carried out on the ZEISS SUPRA 55 (Carl Zeiss, Germany) field-emission scanning electron microscope. AFM was performed on the drop-cast flakes on SiO2 substrates on the Bruker Dimension Icon atomic force microscope (Bruker, USA) using the tapping mode in air. XPS was conducted on the Thermo Fisher ESCALAB 250Xi and X-ray diffraction (XRD) on the D8 Advance with Cu Kα radiation. The UV–vis–NIR absorption spectra were recorded on a Lambda 25 spectrophotometer (PerkinElmer) using Q5-grade quartz cuvettes at room temperature.

2.5. Cell culture and MTT assay

The melanoma A375 cells and cardiomyocyte H9C2 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). The cells were exposed to different concentrations of BP or BP/Bi2O3 nanosheets for different periods of time and the cell viability was determined by the MTT assay.

2.6. Clonogenic assays

The A375 cells (1 × 10³ cells/mL, 2 mL) were seeded on 2 cm petri dish and attached for 24 h. The cells were then incubated with BP/Bi2O3 nanosheets (0.6 μg/mL) for 6 h and then irradiated with or without X-ray radiation (4 Gy). After 6 days of incubation, the cells were stained with Giemsa’s Stain (Solarbio), and the survival fraction of the clones was evaluated.

2.7. Flow cytometry

Flow cytometry was employed to examine the effects of the BP/Bi2O3 nanosheets and X-ray on the A375 cell cycle progression. The A375 cells (2 × 10⁴ cells/mL, 6 mL) were seeded on 6 cm petri dish and attached for 24 h. The cells were then exposed to different concentrations of BP/Bi2O3 nanosheets for 6 h and irradiated with X-ray at 4 Gy on the X-ray linear accelerator for another 24 h. The cells were collected, fixed, and stained with PI in darkness and the cell cycle distribution was analyzed on the Beckman FC500 flow cytometer.

2.8. Annexin V-FITC and PI staining

Annexin V-FITC and PI staining was employed to examine the effects of the BP/Bi2O3 nanosheets and X-ray on the A375 cell in early-stage and late-stage apoptosis cells before cell death. The A375 cells (2 × 10³ cells/mL, 6 mL) were seeded on 6 cm petri dish and attached for 24 h. The cells were then exposed to 2.0 μg/mL of BP/Bi2O3 nanosheets for 6 h and irradiated with X-ray at 4 Gy, and incubated for another 24 h. The cells were collected, and stained with Annexin V-FITC and PI according to the method in manufacturer’s instructions of assay kit (Solarbio).

2.9. Measurement of singlet oxygen generation

Extracellular singlet oxygen generation was examined by photodegradation of 1,3-diphenylisobenzofuran (DPBF). 20 μl/well of the BP nanosheets or BP/Bi2O3 nanosheets were added to 96-well plates at different concentrations and then added to 80 μl/well of DPBF (2 mM) in the solution of BP or BP/Bi2O3 nanosheets. The 96-well plates were immediately analyzed on the microplate (SpectroMaxTM 250) at 410 nm every 2 min for 1 h.

The intracellular singlet oxygen generation also examined by
the DPBF assay. The A375 cells (1 × 10^5 cells/mL, 0.1 mL) were stained with the probe of DPBF (20 μM) for 1 h and seeded on 96-well plates. Different concentrations of BP or BP/Bi2O3 nanosheets were added to the wells and treated with or without X-ray radiation (4 Gy). The 96-well plates were analyzed for singlet oxygen generation in the A375 cells within 1 h using a fluorescence microplate reader (SpectraMax M5, MD, USA) at excitation and emission wavelengths of 410 and 484 nm, respectively.

2.10. Western blot analysis

A375 cells (1 × 10^5 cells/mL, 10 mL) were seeded on 10-cm petri dish and attached for 24 h. The cells were then exposed to 2.0 μg/mL of BP/Bi2O3 nanosheets for 6 h and irradiated with X-ray at 4 Gy, and incubated for another 24 h. Total cellular proteins were extracted by cell lysis buffer from Cell Signaling Technology and determined the protein concentrations by BCA protein assay. The expression levels of proteins associated with DNA damage in A375 cells induced by BP/Bi2O3 nanosheets and X-rays were determined by western blot analysis [46].

2.11. In vivo antitumor activity

The animal studies were approved by the Animal Experimentation Ethics Committee of Jinan University. The A375 cells xenografts were established with about 1 × 10^6 cells in 100 μL serum-free medium injected into the right axilla of male nude mice. After the tumor volume increased to about 35 mm^3, the mice (n = 10) were injected at the caudal vein with 100 μg/kg of BP/Bi2O3 nanosheets for 6 h and the tumor sites of the nude mice were irradiated with X-ray for 4 Gy. The co-treatment was carried out every three days for 7 times. Throughout the 21 days, changes in the tumor volume and body weight were monitored. At the end of the experiments, the tumors of each treatment group were harvested, weighed, fixed with formalin, embedded with paraffin, and sectioned. The tumor inhibition rate was calculated according to the formula: tumor inhibition = (Wc - Wg)/Wc * 100% (Wc and Wg being the tumor weight of the treatment and control group, respectively). The tumor sections underwent H&E staining and TUNEL-Hoechst co-staining. Venous blood was collected from the nude mice eye at the end of the experiments and hematological analysis was conducted to assess the kidney and liver functions of the nude mice.

2.12. MRI detection

Intravoxel incoherent motion diffusion-weighted imaging (IVIM-DWI) was performed on a 3.0-T MR scanner (General Electric, Milwaukee, WI, USA) to observe the hemodynamics, cell density, and activity in living tumor tissues. The pseudocolor signals and quantitative analysis of IVIM-DWI parameters (including standard ADC, slow ADC and fast ADC) were obtained to evaluate the anticancer activity of the different treatment after 21 days.

2.13. Biodistribution of Bi element in mice

To evaluate the tissue biodistribution, the mice were injected with BP/Bi2O3 at 0.5 mg/kg from caudal vein. After 1 day and 28 days, the brain, heart, liver, spleen, and kidney were collected after sacrificed. The concentrations of Bi element could reflect the biodistribution and the clearance of BP/Bi2O3. The collected organs (n = 3) were digested with 0.2 mL of digestion solution containing nitric acid-H2O2 (3:1) for 12 h at 75 °C. Then the solutions were diluted to 10 mL to reduce the acid concentration to lower than 3% (v/v). ICP-MS analysis was applied to determine the amount of Bi element. The standard solution was purchased from Guobiao (Beijing) Testing & Certification Co. Ltd. (Beijing, China). The concentrations of BP/Bi2O3 in organs were represented as percentages of total injected materials.

2.14. Statistical analysis

The experiments were carried out at least three times and all the data were expressed as mean ± standard deviation. Statistical analysis was performed with the SPSS statistical package (SPSS 13.0 for Windows; SPSS, Inc. Chicago, IL) and differences of P < 0.05 (*), P < 0.01 (**) or P < 0.001 (***) were considered statistically significant.

3. Results and discussion

The BP nanosheets are prepared by a modified ultra-sonication liquid exfoliation technique [39]. The transmission electron microscopy (TEM) image in Fig. S1 shows clear edges and wrinkled surfaces revealing high quality. The high-resolution TEM (HR-TEM) image (inset in Fig. S1) clearly shows a lattice spacing of 0.21 nm associated with the (013) plane of BP [47]. The BP/Bi2O3 nanosheets are then synthesized by a hydrolytic method at room temperature according to reactions 1 and 2:

\[
\text{Bi(NO}_3\text{)}_3 + H_2O \rightarrow \text{BiONO}_3 + 2HNO_3 (1)
\]

\[
2\text{BiONO}_3 + H_2O \rightarrow \text{Bi}_2O_3 + 2HNO_3 (2)
\]

In brief, Bi(NO3)3·5H2O is dissolved in ethylene glycol. After added to an NMP solution containing BP nanosheets, Bi(NO3)3 adsorbs onto the nanosheets, especially the defect sites with high reactivity [48]. Residual H2O in the organic solvent reacts with Bi(NO3)3 to form the BiONO3 precipitate (Eq. (1)) which is hydrolyzed to form the Bi2O3 nanoparticles. The residual unhydrolyzed BiONO3 precipitate is discarded after centrifugation. After incorporation of Bi2O3, the Zeta potential of the BP nanosheets in water changes from −27 to +34 mV due to weak ionization of Bi3+. TEM, HR-TEM, atomic force microscopy (AFM), and X-ray diffraction (XRD) are performed to examine the morphology and structure of the BP/Bi2O3 heterostructures. As shown in Fig. 1a and b, the BP/Bi2O3 heterostructures maintain the morphology of the BP nanosheets. The size of the Bi2O3 nanoparticles is 5 ± 3 nm. Fig. 1c shows lattice fringes of 0.33 and 0.20 nm corresponding to the (111) and (023) planes of crystalline Bi2O3, respectively. Fig. 1d and e displays the topography of BP/Bi2O3 heterostructures and the heights of three typical nanosheets are 27.5, 30.1 and 20.7 nm, respectively. According to the statistical TEM and AFM analysis of 100 nanosheets (Fig. 1f and g), the average lateral size of the BP/Bi2O3 heterostructures is 300 ± 80 nm and the average thickness is 25.1 ± 4.5 nm. As shown in XRD spectra in Fig. S2, although the Bi2O3 peaks are weak due to the small concentration on the BP nanosheets, they agree well with the JCPDS No. 41-1449 of the Bi2O3 crystal.

Scanning electron microscopy (SEM), energy-dispersive X-ray spectroscopy (EDS), and X-ray photoelectron spectroscopy (XPS) are employed to determine the composition and chemical states of the BP/Bi2O3 heterostructures. As shown in Fig. 1h and i, the EDS elemental mappings of a BP/Bi2O3 sheet reveal 36.8%, 56.5%, and 6.7% of P, O, and Bi, respectively. The XPS spectra in Fig. 1j confirm the existence of Bi on BP nanosheets. According to the peak area ratios, the molar contents of P, O and Bi are ~35.51%, ~54.99% and ~9.5%, respectively. The XPS is used to measure the elemental composition of the material surface with the test depth usually smaller than 10 nm, thus, the content of Bi is slightly higher than...
the EDS result as the Bi$_2$O$_3$ particles are located on the surfaces of BP sheets. Also, the molar ratio of P and Bi in the hybrid is determined by inductively coupled plasma mass spectrometry (ICP-MS). The results indicate a ratio of about 5:1 (P:Bi), which is close with the EDS results. The high-resolution XPS spectra in Fig. 1k to m are referenced to the C1s peak at 284.5 eV. The peak at 285.8 eV is attributed to $\text{OH}$ of the residual solvent and the bare BP nano-sheets show the typical P2p$_{3/2}$ and P2p$_{1/2}$ doublets at 129.6 and 130.5 eV, respectively, characteristic of crystalline BP [49]. The weak peak at 133.5 eV is due to the oxidized phosphorus (P$_x$O$_y$) sub-bands formed inevitably during processing. The P2p$_{3/2}$, P2p$_{1/2}$, and P$_x$O$_y$ peaks of the BP/Bi$_2$O$_3$ heterostructures shift slightly because of the interaction between BP and Bi$_2$O$_3$ (Fig. 1l).

The two strong peaks at 159.6 and 164.8 eV are associated with Bi 4f$_{7/2}$ and Bi 4f$_{5/2}$ confirming the formation of Bi$_2$O$_3$ (Fig. 1m) [24,50]. The two faint peaks at 157.3 and 162.6 eV are assigned to elemental Bi suggesting that a small portion of Bi ions is reduced [24].

In general, BP lacks stability in air and water [49]. To evaluate the influence of Bi$_2$O$_3$ on the BP stability, the BP and BP/Bi$_2$O$_3$ nanosheets are dispersed in water for 8 days and the optical properties are examined at time intervals of 0, 2, 4, 6, and 8 days. As shown in Fig. 2a, the solution color of the BP nanosheets fades gradually from yellow-brown to colorless but that of the BP/Bi$_2$O$_3$ nanosheets does not change. The corresponding absorption spectra are shown in Fig. 2b and c. During initial dispersion, both the BP and BP/Bi$_2$O$_3$ nanosheets exhibit a typical broad absorption band spanning the ultraviolet and near-infrared regions. However, the absorbance intensity of BP nanosheets in water decreases with time. The absorbance intensity ratio ($A$) at 450 nm decreases by 24.5% compared to the original value ($A_0$) after 1 day and 90% after 8 days. In contrast, the absorption intensity of the BP/Bi$_2$O$_3$ nanosheets is quite stable and only 1% fluctuation in $A/A_0$ is observed during the entire period (Fig. 2d and e). The absorbance decreases because of BP degradation caused by the irreversible oxidation reaction of P atoms forming P$_2$O$_5$ followed by conversion of P$_2$O$_5$ to the final PO$_4^{3-}$ anions [39]. Oxidation tends to occur at defect sites in the BP nanosheets. The enhanced stability of the BP/Bi$_2$O$_3$ nanosheets probably stems from occupation of defect sites on the BP by insoluble Bi$_2$O$_3$ which deter the rapid reaction between BP and oxygen and water. The influence of Bi$_2$O$_3$ on the stability of BP nanosheets is further investigated in humid air (Fig. S3) and enhanced stability is observed as well. In fact, the BP/Bi$_2$O$_3$ powders can be stored in dry air for at least 2 months without obvious degradation. Notably, owing to the high stability of BP/Bi$_2$O$_3$ nanosheets, the morphological integrity and absorbance properties are stabled after X-ray radiation (Fig. S4).

Theoretically, the important action mechanism of radiotherapy is to promote reactive oxygen species (ROS) overproduction in cancer cells to induce DNA damage and cause cell death. ROS are highly reactive ions and free radicals, which mainly include the superoxide anion (O$_2^{-}$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (HO*) and singlet oxygen ($^1$O$_2$). Among these free radicals, $^1$O$_2$ exhibited a relatively longer lifetime and stronger oxidizing ability. Studies have reported that BP promoted $^1$O$_2$ generation under photosensitizing process by energy transfer to ground-state oxygen [37]. The X-ray-PDT properties of the BP/Bi$_2$O$_3$ nanosheets are investigated. Production of $^1$O$_2$ by the BP/Bi$_2$O$_3$ nanosheets under X-ray radiation is assessed using the DPBF fluorescence...
probe as its absorption intensity decreases linearly when oxidized by $^{1}$O$_2$ [37]. As shown in Fig. 3a, b, 60 min after 4 Gy X-ray radiation, the absorption intensity of DPBF decreases by 92.5% for the sample of BP/Bi$_2$O$_3$ nanosheets, whereas under natural illumination decreases by only 40.8%. In addition, we also examined the $^{1}$O$_2$ generation in A375 cells after co-treated with X-ray and BP sheets or BP/Bi$_2$O$_3$ nanosheets by DPBF assay. As shown in Fig. 3c, d, BP/Bi$_2$O$_3$ promoted $^{1}$O$_2$ generation in A375 cells was higher than that of BP sheets, while under the X-ray radiation, both the two nanomaterials enhanced the $^{1}$O$_2$ overproduction in A375 cells. For instance, 10 $\mu$g/mL of BP/Bi$_2$O$_3$ promoted $^{1}$O$_2$ generation at 60 min in A375 cells with about 38.4%, which was much higher than the treatment of BP sheets (about 24.3%). Under X-ray radiation at 4 Gy, 10 $\mu$g/mL of BP/Bi$_2$O$_3$ and BP sheets increased $^{1}$O$_2$ overproduction in A375 cells to about 55.4% and 38.8%, respectively. Therefore, these results suggested that, under X-ray radiation, BP/Bi$_2$O$_3$ nanosheets enhanced $^{1}$O$_2$ overproduction in A375 cells. To further clarify the importance of Bi introduction to enhance the X-ray induced PDT, we have examined other type of Bi-based nanomaterials Bi$_2$Se$_3$ Nanosheets (Bi$_2$Se$_3$ NSs) [51,52] and selenium nanoparticles (SeNPs) [53] for comparison to investigate their X-ray radiation sensitivity. Firstly, we have examined the $^{1}$O$_2$ generation in A375 cells after incubated with SeNPs and Bi$_2$Se$_3$ NSs under X-ray radiation at 4 Gy. As shown in Fig. S5, both SeNPs and Bi$_2$Se$_3$ NSs promoted X-ray radiation-induced $^{1}$O$_2$ overproduction in A375 cells, especially Bi$_2$Se$_3$ NSs. For instance, under X-ray radiation at 4 Gy, Bi$_2$Se$_3$ NSs (10 $\mu$g/mL) promoted $^{1}$O$_2$ generation in A375 cells with about 41.1%, which was higher than the treatment of SeNPs (with about 32.7%). These results further indicated that introduction of Bi can effectively enhance the radiation-induced $^{1}$O$_2$ overproduction. Furthermore, we also examined the $^{1}$O$_2$ generation in A375 cells after treated with BP/Bi$_2$O$_3$ nanosheets and X-ray radiation by DHE assay. As shown in Fig. S6, the free BP/Bi$_2$O$_3$ nanosheets at 10 $\mu$g/mL induced O$_2$ generation at 60 min in A375 cells, and the free X-ray radiation at 4 Gy promoted $^{1}$O$_2$ generation at 60 min in A375 cells with about 12.1%. After co-treated with BP/Bi$_2$O$_3$ nanosheets and X-ray, $^{1}$O$_2$ generation in A375 cells increased to 20.9%, while it still lower than $^{1}$O$_2$ overproduction in A375 cells (reach to 55.4%). These results further indicate $^{1}$O$_2$ as the main type of ROS for BP/Bi$_2$O$_3$-induced X-ray-PDT synergy.

Based on above experimental results, a possible charge separation process for enhanced PDT using BP/Bi$_2$O$_3$ heterostructures under X-ray irradiation is proposed (Fig. S7). It is reported that Bi$_2$O$_3$ has conductive band (CB) and valence band (VB) potentials of about 0.33 and 3.13 eV versus normal hydrogen electrode (NHE), respectively [54]. And the CB and VB of multilayered BP nanosheets are about –0.2 and 0.5 eV versus NHE [55]. Both the CB of Bi$_2$O$_3$ and BP are more positive than the standard redox potentials of O$_2$/O$_2^-$ (0.33 eV versus NHE), thus the X-ray excited electrons in the CB of Bi$_2$O$_3$ and BP cannot reduce O$_2$ to $^{1}$O$_2$. While the X-ray excited holes in the VB of Bi$_2$O$_3$ can still oxidize OH$^-$ to produce $^{1}$OH as the VB of Bi$_2$O$_3$ is more positive than the standard redox potential of OH$^-$/OH (2.4 eV versus NHE). What is more, the CB of Bi$_2$O$_3$ and the VB
of BP are very close and that enable the formation of Z-scheme photosensitized system [56]. When the Bi2O3 and BP are excited with X-ray, the excited electrons in the CB of Bi2O3 quickly recombine with the excited holes in the VB of BP. This increases the chance of energy transfer between the excited electrons in the CB of BP and the surrounding O2, leading to increased O2 and enhanced PDT. Fig. 3e illustrates the proposed mechanism of the BP/Bi2O3 sensitized X-ray-PDT. BP is a direct bandgap semiconductor with good photochemical activity [37] and Bi as a high-Z element absorbs X-ray efficiently [16–18]. When X-ray impacts the BP/Bi2O3 nanosheets, electrons and electron-positron pairs are created due to the photoelectric effect or Compton scattering [7]. Some of the excited electrons induced by BP and Bi2O3 react with surrounding triplet oxygen (3O2) to produce singlet oxygen (1O2). Besides, considering the direct contact between Bi2O3 and BP, it is likely that energy transferred from Bi2O3 to BP can make electrons escape to enhance production of 1O2, which induces cellular damage and apoptosis of nearby cancerous cells.

The efficacy of BP/Bi2O3 sensitized X-ray-PDT is evaluated using A375 cell line. As shown in Fig. 3f, cell viability of A375 cells treated with X-ray radiation at 4 Gy, the A375 cells viability diminishes significantly compared to the control. For instance, the measured viabilities of A375 cells treated with X-ray radiation (4 Gy) only and BP/Bi2O3 nanosheets only are 80.6% and 80.4% respectively. In comparison, the co-treatment shows cell viability to 53.1%, demonstrating that the BP/Bi2O3 nanosheets enhance the anticancer activity of X-ray radiation. Furthermore, we also examined the cell viability of A375 cells after incubation with different nanodrugs, including SeNPs, Bi2Se3 NSs, BP and BP/Bi2O3, with or without co-treatment with X-ray radiation at 2 Gy, 4 Gy and 8 Gy. As shown in Fig. S8, all these nanodrugs significantly enhanced the sensitivity of A375 cells to X-ray radiation. Specifically, the radiosensitization effects of Bi2Se3 NSs was higher than that of SeNPs, while BP/Bi2O3 also exhibited much higher radiosensitization effects than BP nanosheets. To further confirm the X-ray-PDT sensitization effects of BP/Bi2O3, we then evaluated the inhibitory effects of combined treatments on the colony formation and survival fraction of A375 cells. As shown in Fig. 3g and h, under X-ray radiation at 2, 4 and 8 Gy, both BP and BP/Bi2O3 significantly inhibited the cancer cell colony formation, especially the BP/Bi2O3 group. For instance, after co-treatment with BP/Bi2O3 and X-ray radiation at 4 Gy, the survival colony fraction was markedly declined to 13.7%, while the colony formation ratio of BP/Bi2O3 and X-ray radiation alone was found at 90.1% and 61.8%, respectively. In addition, using this colony formation model, we also examined the radiosensitization effects of SeNPs and Bi2Se3 NSs. As shown in Fig. S9, under 4-Gy X-ray radiation, Bi2Se3 NSs significantly inhibited A375 colony formation to 46.4%, which was lower than that treated with SeNPs (about 51.9%), further indicating the enhancement of X-ray-induced PDT by Bi introduction. Therefore, these results suggest that introduction of Bi can effectively enhance the radiosensitization effects of different nanomedicine.
Flow cytometry is implemented to investigate the biological effects of BP/Bi₂O₃ sensitized X-ray-PDT. In most nanomedicine-based cancer therapies, apoptosis and cell cycle arrest are regarded as the major action modes [57]. The sub-G1 and other cell populations are thus measured as indicators for apoptosis and cell cycle arrest, respectively. As shown in Fig. 4a, the sub-G1 peak of the control group is 1.8% and no significant increase can be found from the groups treated with either X-ray (4 Gy) or BP/Bi₂O₃ nanosheets at concentrations of 0.25, 0.5, and 1.0 μg/mL. In contrast, the sub-G1 peaks increase after the co-treatment of X-ray and BP/Bi₂O₃ nanosheets. Specially, the sub-G1 peak of the A375 cells incubated with the BP/Bi₂O₃ nanosheets (1.0 μg/mL) for 6 h and then irradiated with X-ray (4 Gy) increases to 15.2%, that is obviously higher than those of the BP/Bi₂O₃ nanosheets only group (5.5%) and X-ray only group (4.2%). With respect to cell cycle arrest, the G0/G1 phase of the A375 cells treated with either X-ray or BP/Bi₂O₃ nanosheets is almost the same as that of the control group (49.8%). However, for the cells co-treated with X-ray and BP/Bi₂O₃ nanosheets at 0.25, 0.5 and 1.0 μg/mL, the G0/G1 phase increases to 60.8%, 61.0% and 61.2%, respectively, thereby providing evidence that the BP/Bi₂O₃ nanosheets synergistically enhance X-ray-induced apoptosis and cell cycle arrest. Annexin V and PI co-staining assay could be used to discriminate cells at early-stage and late-stage apoptosis after drug treatments. Therefore, we conducted this assay to further explore the mechanisms of BP/Bi₂O₃-enhanced X-ray-induced A375 cell apoptosis. As shown in Fig. 4b, BP/Bi₂O₃ nanosheets significantly enhanced the X-ray-induced cell apoptosis, majorly in late stage. For example, after treated with X-ray and BP/Bi₂O₃ nanosheets alone, the late-stage apoptotic cells were increased from 0.97% (control group) to 11.39% and 13.92%, respectively. Meanwhile, for the cells co-treated with

![Fig. 4.](image-url)
X-ray and BP/Bi2O3 nanosheets, the late-stage apoptotic cells were significantly increased to 33.97%. These results further indicate that BP/Bi2O3 nanosheets synergistically enhance X-ray-induced cancer cell apoptosis.

Generally, singlet oxygen overproduction inside the cells could cause DNA damage and cell death by activating the downstream signal pathways. Therefore, we have examined the expression level of the relevant protein of DNA damage in the treated A375 cells by western blot analysis. As shown in Fig. 4c, the protein levels of p-ATM, p-BRCA1, p-Chk1, p-Chk2 and p-H2A.X (an important biochemical marker of DNA damage) were also up-regulated in A375 cells after co-treated with BP/Bi2O3 nanosheets and X-ray radiation. Taken together these results suggest that BP/Bi2O3 nanosheets enhanced the X-ray-PDT sensitization mainly due to the DNA damage by triggering the singlet oxygen overproduction inside A375 cells. Possible cytotoxicity of the BP/Bi2O3 nanosheets was also examined by Cell counting kit-8 (CCK-8) assay. As shown in Fig. 4d and e, BP/Bi2O3 nanosheets have little cytotoxicity in melanoma A375 cells and normal cardiomyocyte H9C2 cells. Their cytocompatibility for normal H9C2 cells are even better than that of the bare BP nanosheets, due to that Bi2O3 suppresses the rapid reaction between BP and surrounding molecules.

Melanoma xenografts nude mice are used as the animal model to assess the in vivo cancer therapeutic efficacy of the BP/Bi2O3 nanosheets as radiosensitizers. 100 μg/kg of the BP/Bi2O3 nanosheets are intravenously injected to the mice 6 h before exposure to X-ray radiation (4 Gy) on a clinical radiotherapeutic instrument. As shown in Fig. 5a–c, the BP/Bi2O3 + X-ray group show much more reduced tumor weight than the other groups, including the BP/Bi2O3 only group (without X-ray), X-ray only group (without BP/Bi2O3), and control group. Typically, the tumor weight of the BP/Bi2O3 only group is 1.045 g which is close to the control group of 1.065 g, demonstrating no obvious tumor inhibition effects of the BP/Bi2O3 itself. In contrast, after co-treatment with BP/Bi2O3 + X-ray, the tumor weight diminishes significantly to 0.365 g which is much less than that of the X-ray only group (0.623 g). Correspondingly, the tumor inhibition rate of the BP/Bi2O3 + X-ray co-treatment group reaches 65.6% that is higher than that of the X-ray only group (41.2%). The enhanced tumor inhibition effects rendered by the BP/Bi2O3 + X-ray co-treatment are clearly demonstrated by tumors stripped from the different treatment groups as illustrated in Fig. 5d. Furthermore, there are no significant

Fig. 5. In vivo cancer radiotherapy: (a) Changes in tumor volume, (b) tumor weight, and (c) tumor inhibition ratio of melanoma xenografts in nude mice after intravenous injection of BP/Bi2O3 nanosheets and/or X-ray radiation for 21 days (n = 10); (d) Photographs of tumors stripped from different treatment groups; (e) H&E staining and TUNEL-Hoechst co-staining assay of tumor tissues from different treatment groups; (f) Pseudocolor signals and (g) quantitative analysis of IVIM-DWI parameters of tumor tissues in different treatment groups by MRI analysis.
changes in the body weight after different treatments (Fig. S10) and the results indicate that the BP/Bi2O3 nanosheets enable synergistic cancer radiotherapy in vivo.

The cell morphology and apoptosis of tumor tissues are analyzed (H&E staining). According to hematoxylin and eosin staining, the BP/Bi2O3 nanosheets + X-ray co-treatment decreases the nuclear abnormality, abnormal mitoses, and microvascular formation, indicating inhibited tumor malignancy. TUNEL-Hoechst co-staining is further utilized to evaluate the apoptotic characteristics of each treatment group (Fig. 5e). Compared to other groups, the BP/Bi2O3 nanosheets enhance X-ray-induced apoptotic DNA fragmentation as evidenced by the significant increase in green fluorescence. All in all, these results confirm that the BP/Bi2O3 sensitized radiotherapy induces tumor inhibition by triggering tumor cell apoptosis.

Intravoxel incoherent motion diffusion-weighted imaging (IVIM-DWI) of magnetic resonance (MR) was further used to observe the hemodynamics and activity in tumor tissues. As shown by the MR images taken to investigate the anticancer activity of BP/Bi2O3 induced X-ray-PDT in vivo (Fig. 5f and g), increases in the standard/slow ADC values and decreases in the fast ADC values suggest that the BP/Bi2O3 + X-ray co-treatment enhances necrosis and reduces the blood flow in tumor tissues compared to the BP/Bi2O3 or X-ray only groups. The possible toxicity of the BP/Bi2O3 nanosheets and X-ray radiation in the main organs including the heart, liver, spleen, lung, and kidney, is examined by H&E staining. As shown in Fig. 6a, there are no obviously pathological changes in these major organs after treatment with the BP/Bi2O3 and/or X-ray for 21 days. The hematological analysis is employed to examine the effects of each treatment on the functions of kidney and liver of the nude mice. As shown in Fig. 6b, the kidney function revealed by blood indexes of uric acid (URIC) and urea (UREA), kidney function revealed by blood indexes of uric acid (URIC) and urea (UREA) and liver function related to alanine aminotransferase (ALT), and low-density lipoprotein (LDL-c) in healthy, A375 tumor-bearing, BP/Bi2O3 treated, and BP/Bi2O3 + X-ray co-treated nude mice.

4. Conclusions

In summary, the BP/Bi2O3 heterostructures are synthesized and used as new radiosensitizers for synergistic cancer radiotherapy. The Bi2O3 decoration inhibits the rapid degradation of BP nanosheets by occupation of the defect sites, thus the BP/Bi2O3 heterostructures show excellent stability in water. The synergistic effects rendered by Bi2O3 and BP trigger overproduction of \(^{1}O_2\) producing efficient X-ray-PDT effect to induce cell apoptosis and cell cycle arrest. In vivo treatment of melanoma conducted on a clinical radiotherapeutic instrument demonstrates that the BP/Bi2O3 sensitized radiotherapy inhibits tumor growth efficiently. Furthermore, the BP/Bi2O3 heterostructures composed of biological friendly P, O, and Bi elements show excellent biocompatibility in vitro and in vivo. The BP-based radiosensitizer thus has immense clinical potential for synergistic cancer radiotherapy and our findings reveal a general strategy to fabricate stable BP-based heterostructures suitable for different applications.

Author contributions

The manuscript was written through contributions of all authors.
References


Supporting Information

Stable Black Phosphorus/Bi₂O₃ Heterostructures for Synergistic Cancer Radiotherapy

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Fig. S1. TEM and HR-TEM (inset) images of BP nanosheets.
Fig. S2. XRD spectra of BP and BP/Bi$_2$O$_3$ nanosheets.
Fig. S3. Stability of BP and BP/Bi$_2$O$_3$ nanosheets in air with humidity of about 95%.
Fig. S4. (a) Absorption spectra and (b) TEM image the BP/Bi$_2$O$_3$ nanosheets with X-ray radiation.
**Fig. S5.** SeNPs and Bi$_2$Se$_3$ NSs (10 µg/mL) promote radiation-induced singlet oxygen overproduction in A375 cells.
Fig. S6. BP/Bi$_2$O$_3$ nanosheets promotes radiation-induced superoxide anion (O$_2^•$) generation in A375 cells.
Fig. S7. Z-scheme enhanced PDT using BP/Bi$_2$O$_3$ heterostructures under X-ray irradiation.
Fig. S8. (a) The cell viability of A375 cells after incubated with different concentration of BP and BP/Bi$_2$O$_3$ nanosheets, SeNPs and Bi$_2$Se$_3$ NSs. (b, c, and d) A375 cell viability after incubated with different drugs and under the X-ray radiation at 2 Gy, 4 Gy and 8 Gy.
Fig. S9. SeNPs and Bi$_2$Se$_3$ NSs (0.6 µg/mL) enhance the inhibitory effects of X-ray at varied radiation doses on the colony formation and survival fraction of A375 cells.
**Fig. S10.** Changes in body weight of melanoma xenografts bearing nude mice after intravenous injection of BP/Bi$_2$O$_3$ nanosheets and/or X-ray radiation for 21 days ($n$ = 10).
Fig. S11. The biodistribution of Bi element in the injected mice after 1 day and 28 days.